Rapid Multiplex PCR and Real-Time TaqMan PCR Assays for Detection of *Salmonella enterica* and the Highly Virulent Serovars Choleraesuis and Paratyphi C

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*Salmonella enterica* is a human pathogen with over 2,500 serovars characterized. *S. enterica* serovars Choleraesuis and Paratyphi C are two globally distributed serovars. We have developed a rapid molecular-typing method to detect serovars Choleraesuis and Paratyphi C in food samples by using a comparative-genomics approach to identify regions unique to each serovar from the sequenced genomes. A *Salmonella*-specific primer pair based on *oriC* was designed as an internal control to establish accuracy, sensitivity, and reproducibility. Serovar-specific primer sets based on regions of difference between serovars Choleraesuis and Paratyphi C were designed for real-time PCR assays. Three primer sets were used to screen a collection of over 100 *Salmonella* strains, and both serovars Choleraesuis and Paratyphi C gave unique amplification patterns. To develop the technique for practical use, its sensitivity for detection of *Salmonella* spp. in a food matrix was determined by spiking experiments. The technique was also adapted for a real-time PCR rapid-detection assay for both serovars Choleraesuis and Paratyphi C that complements the current procedures for *Salmonella* sp. isolation and serotyping.

*S. enterica* is the causative agent of a wide range of acute serovar-specific infections, which include gastroenteritis (*S. enterica* serovar Typhimurium) and bacteremia (*S. enterica* serovar Choleraesuis and *S. enterica* serovar Typhi) (11, 20, 29, 30). *S. enterica* is transmitted to humans via the food chain, leading to morbidity and mortality, and can also cause severe economic losses due to food recalls (4, 26, 29, 32, 33). It is estimated that *S. enterica* infections cause 1.4 million cases and cost between $500 million and $2.3 billion in losses in the United States per year, and this is only a fraction of the incidence rate in developing countries (18, 21, 33).

The genus *Salmonella* currently contains two species, *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V) (13–16, 19, 22, 25). *S. enterica* is subdivided into seven subspecies: I, II, IIIa, IIIb, IV, VI, and VII (3, 22). *Salmonella* infections in warm-blooded animals are generally caused by strains from *S. enterica* subspecies I. Currently, there are more than 1,400 serovars within *S. enterica* subspecies I (22). Certain serovars cause variable disease symptoms in different hosts, ranging from gastroenteritis to highly invasive diseases. For example, *S. enterica* serovar Choleraesuis causes sepsis or extraintestinal focal infection in humans and paratyphoid in swine (6). *S. enterica* serovars Choleraesuis and Paratyphi C have higher mortality rates in humans than other *Salmonella* serovars (5, 6, 11). *S. enterica* serovar Paratyphi C is highly adapted to humans, in whom it can cause a typhoid-like disease and recurrent intra-abdominal abscesses (9, 28). Interestingly, there has been a sharp increase in the number of multidrug-resistant outbreaks associated with both serovars Choleraesuis and Paratyphi C in recent years (9, 28).

Serotyping is the method of choice to identify and discriminate isolates of *S. enterica*. However, the method has a number of deficiencies, including the inability to serotype between 5 and 8% of isolates and incorrect typing due to the loss of surface antigens (12, 27). There has been a general move toward molecular methods of *Salmonella* detection and typing, which are based less on phenotypic features and more on stable genotypic characteristics (1, 12). Molecular methods, such as PCR, are used for the identification of many food pathogens. Real-time (RT) PCR shows promise as an effective and accurate technology and has a high degree of agreement with conventional culture methods for *Salmonella* (10, 31). Whole-genome *Salmonella*-sequencing projects have shown that there is extensive sequence conservation and synteny among genomes (1, 12, 17, 23). It is estimated, however, that an average of 10 to 20% unique DNA has been acquired among the different serovars since the species diverged (1, 12, 17, 23). Comparative microarray analysis of the closely related serovars Enteriditis, Gallinarum, and dublin identified specific regions unique to each serovar (23, 24).

The detection and accurate identification of *S. enterica* pathogens in the food chain at various stages (from field to fork) is essential for the prevention of a wide variety of life-threatening infections. In this study, we performed in silico *S. enterica* whole-genome sequence comparisons to identify regions unique to two highly virulent *S. enterica* serovars, Choleraesuis and Paratyphi C. In addition, we designed a primer

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pair based on the oriC region that specifically amplifies Salmonella isolates only. A multiplex PCR protocol was developed using three primer sets for the detection of serovars Choleraesuis and Paratyphi C. This was further developed into a rapid RT-PCR TaqMan assay to detect these serovars in a food matrix.

**MATERIALS AND METHODS**

**Bacterial strains.** Salmonella Reference Collection B (SARB), encompassing 37 serovars of subspecies I, and Salmonella Reference Collection C (SARC), encompassing 80 isolates of S. enterica and S. bongori, were used for primer pair validation (see Table S1 in the supplemental material) (2, 3). A total of 38 S. enterica strains recovered in Ireland representing nine serovars were also included in the study (see Table S1 in the supplemental material). The total collection of isolates examined included 190 Salmonella strains. Overnight cultures were prepared in Luria-Bertani (LB) medium supplemented with 2% NaCl at 37°C with agitation. The strains were stored at −70°C in LB broth containing 20% glycerol. Strains were chosen that encompassed different subspecies, as well as the species S. bongori, to validate our Salmonella indicator primer set for the oriC sequence. For the validation of serovar detection, 110 strains representing 56 serovars were chosen to be screened, including strains that are highly similar genetically (2, 3). Strains of Escherichia coli, Pseudomonas fluorescens, and Listeria monocytogenes were included in the screening process as negative controls.

**Bioinformatic analysis.** The full nucleotide sequences and annotations of S. enterica serovar Typhimurium LT2 (NC_003197), S. enterica serovar Typhi CT18 (NC_003196), S. enterica serovar Typhi Ty2 (NC_004631), S. enterica serovar Choleraesuis SC-B67 (NC_006905), and S. enterica serovar Paratyphi A ATCC 9190 (NC_006511) were downloaded from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). The Web-based Artemis Comparison Tool (WebACT) (http://www.webact.org/WebACT/home) was used to identify regions unique to each serovar. The Basic Local Alignment Search Tool (BLAST) (http://ncbi.nlm.nih.gov/BLAST) was used to check for sequence homology in the database.

**Genomic DNA isolation.** Genomic DNA was isolated using a Gnome DNA isolation kit (Bio 101 Inc., La Jolla, CA) according to the manufacturer’s instructions. The extracted genomic DNA was resuspended in 300 μl of Tris-EDTA, Primer3 (v.0.4.0) was used to design DNA primer pairs and DNA probes. The primer and probe outputs were manually adjusted for incorporation into a multiplex PCR assay. The primer pairs for multiplex PCR (mPCR) were ConOri-F (GCCGTGTTGCTCTACTAAC) and ConOri-R (AGAAGCGGAACTGAAAAG), which amplify a 461-bp product; CsPcSC4352-F (TACCAAGCAGGTTAAAACAGTGGG) and CsPcSC4352-R (TATACGGACCA); 0.2 μM of RTSTM3664-F (GAATCATGCCGGTACGCG) and RTSTM3664-R (GCGGTGGATTCTACTCAAC) and ConOri-R (AGAAGCGGAACTGAAAAG), which amplify a 709-bp product; and STMS664-F (ATGAAAATCCTGGCGCTTGGCTC) and STMS664-R (TACTAGAGGGCGGCCAATGGA), which amplify a 997-bp product.

**Serovar detection assay.** Spiked samples were prepared as follows. Single colonies were inoculated into 5 ml of LB broth and incubated at 37°C for 6 h. Serial dilution and plating were used to determine bacterial CFU. For the comminuted food matrix, 10 g of ground meat (or 10 ml of pasteurized milk product) was inoculated with 1 ml of spiked LB broth. Preenrichment was carried out by stomaching 10 g of the spiked matrix with 90 ml of buffered peptone water (Oxoid) supplemented with 1.6 ml of 0.1% Novobiocin (Sigma) for 30 s and incubating at 37°C for 18 h (7). After the incubation, 0.1 ml of the culture was inoculated into 10 ml of Rappaport-Vassiliadis (RV) medium (Oxoid) and incubated at 42°C for 24 h. In tandem with this, 1 ml of the culture was inoculated into 10 ml of Muller-Kaufmann tetrahionate Novobiocin broth (MKtn) (Cruinn Diagnostics Ltd., Dublin, Ireland) and incubated at 37°C for 24 h. The presence of presumptive positive Salmonella spp. was confirmed on xylose lysine deoxycholate agar (Cruinn) and on Haraquein Salmonella ABC agar.

**mPCR assay.** The mPCR assay was conducted on 1 μl of the enrichment media from spiked samples or 0.75 μl of DNA. The PCR cocktails each included 5 μl of 5× buffer, 1.5 mM MgCl₂, 125 μM nucleotide mixture, 15 pmol of each primer, 1 U of Taq polymerase, and sterile distilled water to 50 μl. A Peltier thermal cycler 200 was used for all PCRs. The PCR conditions used were 96°C for 5 min (or 2 min for initial DNA development), followed by 25 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min.

**RT-PCR assay.** RT-PCR was conducted using the Quantitect Multiplex PCR kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, with the following adaptations. The cycling conditions were amended to 95°C for 15 min, followed by 30 cycles of 94°C for 15 s and 60°C for 60 s. The reaction mixture contained 0.2 μM of RSTM3664-F (GAATCATGCCGGTACGCG) and RSTM3664-R (GCGGTGGATTCTACTCAAC), 0.4 μM of RTSC4352-F (ATAAAGATCTAGGTCCTA), RTSC4352-R (TATAAAGTCTAAGCTGTTGCTCA), and RTConOr-R (CGGATCCTGATTTGTTGCTGA-BHQ-3) and RTConOri-R (GCGGTGGATTCTACTCAAC), which amplify a 461-bp fragment; and 5 μl of template made up to 50 μl with sterile distilled water. The TaqMan RT-PCR was conducted on a Rotor-Gene 6000 (Corbett Life Science, Sydney, New South Wales, Australia). The copy numbers of the target identification sequences were calculated using the following formula: \( m = (r) \times 1.986 \times \frac{n}{2} \) where \( n \) is the number of base pairs and \( m \) is the mass of the DNA.

**RESULTS AND DISCUSSION**

**Design of an IAC.** The Salmonella origin of replication (oriC) was chosen as a genus-specific region for an internal amplification control (IAC) to establish the specificity and sensitivity of the mPCR. The IAC primer pair was validated using 72 subspecies I strains and 80 strains encompassing subspecies II, IIIa, IIIb, IV, and VI and S. bongori (see Table S1 in the supplemental material) (2, 3). The oriC primer pair (ConOri-F and ConOri-R) amplified an expected 461-bp PCR product in all Salmonella strains tested, and no product was obtained with the negative controls (Fig. 1).

**Identification of serovar-specific genomic regions.** Comparative genomic analysis identified regions unique to S. enterica serovars Choleraesuis and Paratyphi C. The genomic analysis tool WebACT was used to compare the genome of serovar Choleraesuis to the published sequenced genomes of S. enterica serovars Typhimurium LT2, Paratyphi A ATCC 9150, Typhi CT18, and Typhi Ty2. This analysis identified a 12.8-kb region unique to serovar Choleraesuis between open reading frames SC4343 and SC4353, which was previously identified as a metabolic island (6). In addition, an 11-kb region encompassing open reading frames STMS664 to STMS674 was identified that was present only in serovars Paratyphi C and Typhimurium. A PCR primer set (CsPcSC4352-F and CsPcSC4352-R) was designed for the 12.8-kb region and was used to screen our strain collection. An expected 461-bp PCR product was present in all serovar Choleraesuis isolates tested; however, a product was also present in serovar Paratyphi C isolates. Next, we designed primer pair STMS664-F and STMS664-R specific to the 11-kb region and used it to screen the strain collection. An expected 461-bp product was obtained from all serovar Paratyphi C isolates, as well as from all serovar Typhimurium, Enteriditis, and Typhi isolates, but was absent from all serovar Typhi isolates.
Choleraesuis isolates examined. Both primer pairs were chosen as potential serovar-specific probes using an mPCR approach.

**Development and optimization of the mPCR screen.** To develop the mPCR assay, primers ConOri-F and ConOri-R, CsPcSC4352-F and CsPcSC4352-R, and STM3664-F and STM3664-R were combined and conditions were optimized as described in Materials and Methods to achieve specific and sensitive amplification of the three target regions. The mPCR assay was validated against the strain collection to detect all *Salmonella* isolates and serovars Choleraesuis and Paratyphi C when present (Fig. 2A). The positive identification of all *Salmonella* isolates resulted in the amplification of a 461-bp PCR product (Fig. 2A). From serovar Choleraesuis isolates, two PCR products were amplified, a 461-bp *Salmonella*-specific product and a 709-bp *serovar Choleraesuis*-specific product. From serovar Paratyphi C isolates, three PCR products were amplified: 461 bp, 709 bp, and 986 bp (Fig. 2A). All isolates of serovars Enteriditis, Typhimurium, and Typhi tested gave the predicted amplicon patterns, resulting in 100% accuracy for 152 strains tested (see Table S1 in the supplemental material).

**Adaptation of the mPCR for analysis of food samples.** For practical use, the mPCR was adapted to methodologies for the identification of the pathogens in food matrices. To ensure compliance with international regulations, the current international standard of operation, ISO 6579:2002, was taken into consideration. We included two individual enrichment steps to improve sensitivity. Food matrices spiked with *Salmonella* were preenriched in buffered peptone water medium, followed by enrichment in RV and MKTTn media, and the mPCR assay was conducted directly on the enrichment media. The expected 461-bp *Salmonella*-specific PCR band; the 461-bp and 709-bp bands for serovar Choleraesuis; and the 461-bp, 709-bp, and 986-bp PCR bands specific for serovar Paratyphi C were produced (Fig. 2B). However, MKTTn medium resulted in fainter bands and, in the serovar Typhimurium sample, the absence of an amplicon, whereas the mPCR results from the RV medium gave banding patterns identical to those from the assay conducted on DNA (Fig. 2). The MKTTn medium itself may inhibit the reaction or may contain a higher level of food matrix inhibitory factors, since 1 ml of spiked matrix was added to MKTTn medium as opposed to 0.1 ml in RV medium. Our method showed the identification of *Salmonella* directly from media with no requirement for prior DNA isolation. The specificity was also investigated using food matrices spiked with several other prevalent food-borne pathogens: serovar Typhimurium, *E. coli*, *P. fluorescens*, and *L. monocytogenes* (Fig. 2B).

In each case, the assay identified only *Salmonella* spp. and *serovar Choleraesuis* and Paratyphi C isolates, and no amplicon was detected from non-*Salmonella* isolates.

To calculate sensitivity, food matrices were spiked with different levels of each of the serovars of interest, serovars Choleraesuis and Paratyphi C, from RV medium. An overnight culture of serovar Choleraesuis was diluted to 250 CFU, 25 CFU, 3 CFU, and extinction (the point at which there were no longer culturable colonies present in the dilution). Each of the dilutions was used to spike the matrix. Enrichment and mPCR assays were conducted directly on the RV medium. Serovar Choleraesuis was identified by two specific 461-bp and 709-bp bands at 250 CFU and 25 CFU (Fig. 3A, lanes 1 and 2, respectively). However, the assay could not detect bacteria at the level of 3 CFU (Fig. 3A, lane 3). There was also no amplification in the dilution to extinction or in the negative control. An overnight culture of serovar Paratyphi C was diluted to 200 CFU, 20 CFU, 2 CFU, and extinction. Serovar Paratyphi C was identified by the amplification of three specific PCR bands of 461 bp, 709 bp, and 986 bp at 200 CFU, 20 CFU, and 2 CFU (Fig. 3B, lanes 1 to 3, respectively). Faint bands of the appropriate size for serovar Paratyphi C identification were present at extinction, and the unspiked food matrix sample had a single band indicating a member of the genus *Salmonella* present in the food matrix prior to the spiking. This was not too surprising, as up to 10% of food matrices can be contaminated with *Salmonella* (8). PCR contamination was ruled out, as no amplicon was present in the control lane containing no template (lane 6). Our results show that the mPCR assay detects each serovar at very low levels (Fig. 3A and B).

**Reproducibility in different food matrices.** To ensure the robustness and reproducibly of the assay, and also to assess interference by the matrix itself or its normal bacterial flora, an alternative food matrix, milk, was tested. This was done by
spiking pasteurized milk in the same manner as described for comminuted meat. *Salmonella* and both serovars Paratyphi C and Choleraesuis could be clearly identified from the milk matrix by their specific banding patterns described above (Fig. 3C). The natural bacteria in the matrix, as well as the matrix itself, did not interfere with the identification of *Salmonella* or the serovars, and no inhibition was seen.

### TaqMan RT-mPCR validation from DNA sources

A TaqMan RT-mPCR assay was subsequently developed for the identification of *Salmonella* spp. and the serovars Choleraesuis and Paratyphi C. This adaptation made the assay more automated and rapid. Three different fluorescent dyes were used, and probes and fluorophore information are detailed in Table 1.

Standard curves were initially created for the calculation of the thresholds. Each copy number dilution was tested in duplicate for accuracy and reproducibility and was found to be almost linear, with the $R^2$ values being close to 1.0 (range, 0.97, 0.99, 0.99). The amplification efficiencies for the red and green channels were in the range of 90 to 100% (98% for both); this would show a near doubling of the amplicon in each cycle. The reaction efficiency value for the orange channel was 89%, which is lower than the cutoff point. However, as the assay is used for identification purposes, not quantification purposes, this is an accepted value. The slope on which the reaction efficiency was calculated was between −3.1 and −3.6 (with −3.322 corresponding to 100% PCR efficiency); all of the assay values lay within this range. With the test samples of DNA, all of the *Salmonella* spp. were identified by probes designed for the oriC sequence (orange channel). The green channel measured the fluorescence of the probe designed for a region within SC4343 to SC4353, which identifies serovars Choleraesuis and Paratyphi C among all the other serovars. The red channel measured the fluorescence of the probe designed for a region within STM3664 to STM3674, which differentiates serovars Choleraesuis and Paratyphi C from each other. Fig. S1A in the supplemental material shows that each of the *Salmonella* isolates tested was identified as positive, that both of the serovars of interest were also identified, and that no nonspecific fluorescence was detected.

### Adapting the TaqMan RT-mPCR for practical use

For practical use, the DNA RT-mPCR-based methodology was tested for detection of bacteria in food matrices, taking into consideration international standards of operation. Figure S1B and C in the supplemental material shows the assay conducted directly on the enrichment media. *Salmonella* spp. and serovars Choleraesuis and Paratyphi C were clearly identified. The comminuted food matrix of turkey meat was spiked with a number of other bacteria, *S. enterica* serovar Typhimurium (7 × $10^9$ CFU/ml), *E. coli* (8 × $10^9$ CFU/ml), *P. fluorescens* (1 × $10^9$ CFU/ml), and *L. monocytogenes* (2 × $10^9$ CFU/ml) (see Fig. S1B and C in the supplemental material). The fluorescence with the samples spiked with *P. fluorescens* and *L. monocytogenes* was equivalent to that of the negative controls. The *E. coli* sample gave a degree of fluorescence identified by the orange channel; however, the adjustment of the threshold made it clear which of the samples were *Salmonella* positive and which were negative. *S. enterica* serovar Typhimurium was identified as a member of the genus *Salmonella*. To ensure that there was no contamination, two negative controls were tested: a reaction tube with no template and another that was from media with no initial spike. Both of these samples gave no

### Table 1. Probe and primer information, including fluorophore details, for the TaqMan RT-PCR assay conducted on a RotorGene 6000

<table>
<thead>
<tr>
<th>Probe designation</th>
<th>Channel</th>
<th>F/O &quot;(5'3')&quot;</th>
<th>Wavelength (nm)</th>
<th>Amplicon size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTConOri-Pr</td>
<td>Orange</td>
<td>ROX/BHQ-2</td>
<td>575</td>
<td>602</td>
</tr>
<tr>
<td>RTSC4352-Pr</td>
<td>Green</td>
<td>FAM/BHQ-1</td>
<td>488</td>
<td>518</td>
</tr>
<tr>
<td>RTSTM3664-Pr</td>
<td>Red</td>
<td>Cy5/BHQ-3</td>
<td>649</td>
<td>670</td>
</tr>
</tbody>
</table>

*a* F, fluorophore; *Q*, Black Hole (BH) quencher; FAM, 6-carboxyfluorescein. 

**nt**, nucleotides.
fluorescence in any channel. Importantly, the natural bacterial flora and the proteins in the matrix itself did not interfere with the RT-PCR assay. Although the assay functioned as expected when conducted on the bacteria in RV samples, the crossing point (cycle threshold) was slightly higher than when the assay was conducted on pure DNA.

This highly reproducible, specific, and sensitive assay has the ability to detect two closely related yet pathogenically distinct serovars. These results demonstrate that Salmonella spp. can be identified with great speed, accuracy, and reproducibility. Currently, Salmonella spp. require up to 5 to 7 days for identification and Salmonella serovar identification can take up to 3 weeks; our assay identifies Salmonella spp. and the serovars of interest in less than 2 days (46 h). This study will complement the current lengthy procedures for Salmonella confirmation and serotyping and emphasizes the advantages of utilizing bioinformatics for beneficial practical applications.

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The mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

REFERENCES


